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(54) Use of 17-beta-hydroxywortmannin and analogs thereof for phosphatidylinositol-3-kinase.

(57) 17 α -hydroxywortmannin and certain of its analogs are inhibitors of phosphatidylinositol 3-kinase. The compounds are particularly useful for inhibiting phosphatidylinositol 3-kinase in mammals and for treating phosphatidylinositol 3-kinase-dependent conditions, especially neoplasms, in mammals.

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The present invention relates to a method of inhibiting phosphatidylinositol 3-kinase (PI 3-kinase) in a lysed or whole cell by contacting the lysed or whole cell with a compound known as 17 β -hydroxywortmannin or one of certain 17 β -hydroxywortmannin analogs. Such compounds also can be used to selectively inhibit phosphatidylinositol 3-kinase in mammals, particularly humans, and to treat phosphatidylinositol 3-kinase-dependent conditions, particularly neoplasms, in humans.

The metabolism of inositol phospholipids is believed to be an essential part of the receptor-mediated signal transduction pathways in response to various hormones and growth factors [see, e.g., Berridge, M.J., *et al.*, *Nature*, **312**: 315-321 (1984); Nishizuka, Y., *Science*, **225**: 1365-1370 (1984)].

In this signaling pathway, two intracellular second messengers, inositol 1,4,5-trisphosphate and diacylglycerol are generated through the hydrolysis of phosphatidyl 4,5-bisphosphate by phospholipase C. Inositol 1,4,5-trisphosphate releases Ca²⁺ from intracellular Ca²⁺ stores leading to the activation of Ca²⁺/calmodulin-dependent kinase; diacylglycerol activates protein kinase C. Following breakdown, phosphatidylinositol 4,5-bisphosphate is rapidly resynthesized by stepwise phosphorylation of phosphatidylinositol by phosphatidylinositol 4-kinase and phosphatidylinositol-4-phosphate kinase. These 2 kinases appear to play important roles in the production of second messengers (see, e.g., Duell, T.F., US Pat. No. 5,001,064 (1991); Shibasaki, F., *et al.*, *J. Biol. Chem.*, **266** (13): 8108-8114 (1991)).

More recently, the existence of another phosphatidylinositol kinase has been identified and associated with certain activated tyrosine kinases [Courtneidge, S.A., *et al.*, *Cell*, **50**: 1031-1037 (1987); Kaplan, D.R., *et al.*, *Cell*, **50**: 1021-1029 (1987)]. This kinase, identified as phosphatidylinositol 3-kinase has been found to phosphorylate the 3-position of the inositol ring of phosphatidylinositol (PI) to form phosphatidylinositol 3-phosphate (PI-3P) [Whitman, D., *et al.*, *Nature*, **332**: 664-646 (1988)].

In addition to PI, this enzyme also can phosphorylate phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate to produce phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (PIP₃), respectively [Auger, K.R., *et al.*, *Cell*, **57**: 167-175 (1989)].

PI 3-kinase physically associates with tyrosine kinases such as pp60^{src}, polyoma middle T/pp60^{src}, platelet-derived growth factor receptor, colony stimulation factor-1 receptor, and insulin receptor (see, e.g., Shibasaki *supra*), suggesting it has important, but yet undefined roles in signal transduction, mitogenesis, cell transformation, and other cellular events involving protein tyrosine kinases that associate with and activate PI 3-kinase. PI 3-kinase activity also has been identified in association with G-protein receptors in neutrophils and platelets in neutrophils [Traynor-Kaplan, A.E., *et al.*, *Nature*, **334**: 353-356 (1988); and Mitchell, C.A., *et al.*, *Proc. Nat. Acad. Sci.*, **87**: 9396-9400 (1990)]. However, activation of PI 3-kinase in the neutrophil occurs independently of tyrosine phosphorylation [Vlahos, C.J., *et al.*, *FEBS Letters*, **309**(3): 242-248 (1992)].

PI 3-kinase exists as a tightly associated heterodimer of an 85 kDa regulatory subunit and an 110 kDa catalytic subunit, and is found in cellular complexes with almost all ligand-activated growth factor receptors and oncogene protein tyrosine kinases [Cantley, L.C., *et al.*, *Cell*, **64**: 281-302 (1991)]. The 85 kDa regulatory subunit apparently acts as an adaptor protein which allows the 110 kDa catalytic subunit of PI 3-kinase to interact with growth factor receptors and tyrosine phosphorylated proteins [Margolis, C., *Cell Growth Differ.*, **3**: 73-80 (1992)].

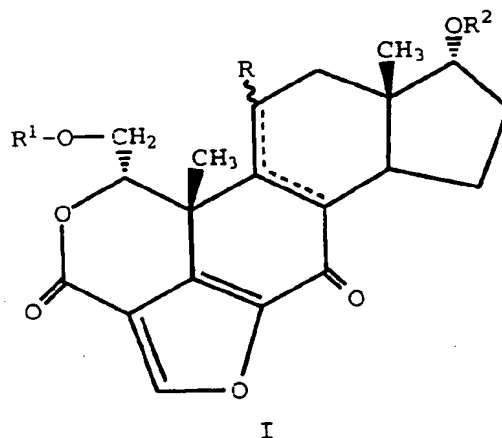
Although PI 3-kinase appears to be an important enzyme in signal transduction, with particular implications relative to mitogenesis and the malignant transformation of cells, only a limited number of compounds have been identified as having inhibitory activity against PI 3-kinase [see, e.g., Matter, W.F., *et al.*, *Biochem. Biophys. Res. Commun.*, **186**: 624-631 (1992)]. Contrary to the selective PI 3-kinase activity of the compounds used in the methods of the present invention, the bioflavonoid compounds used by Matter, *et al.*, particularly quercetin and certain analogs thereof, inhibit PI 3-kinase and other kinases such as protein kinase C and PI 4-kinase (Matter, *et al.*, *supra*).

Thus, the present invention provides a method for inhibiting phosphatidylinositol 3-kinase in a lysed or whole cell with 17 β -hydroxywortmannin or one of certain 17 α -hydroxywortmannin analogs.

The present invention also provides a method for inhibiting phosphatidylinositol 3-kinase in mammals, particularly humans, using 17 β -hydroxywortmannin or one of certain analogs thereof.

Furthermore, the present invention provides a method for treating phosphatidylinositol 3-kinase-dependent conditions, particularly neoplasms, in mammals.

The present invention provides a method for inhibiting phosphatidylinositol 3-kinase in a lysed or whole cell comprising contacting a lysed or whole cell with a compound of formula I



wherein

R is H or acetoxy;

R¹ is C₁-C₈ alkyl;

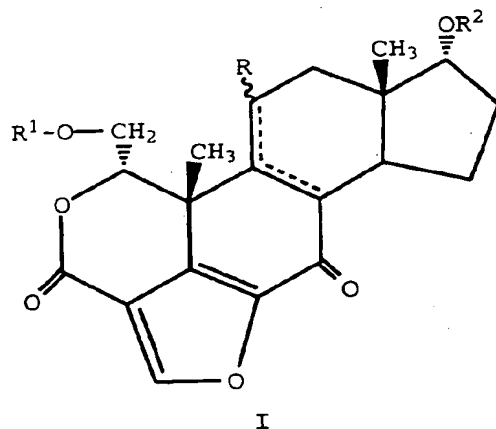
R² is hydrogen, C₁-C₈ alkyl, or a group of the formula -COOR³; and

R³ is H or C₁-C₈ alkyl.

The present invention also provides a method for inhibiting phosphatidylinositol 3-kinase in a mammal comprising administering to said mammal a phosphatidylinositol 3-kinase inhibiting amount of a compound of formula I.

The present invention further provides a method for treating a phosphatidylinositol 3-kinase-dependent condition in a mammal in need of such treatment comprising administering to said mammal a phosphatidylinositol 3-kinase inhibiting amount of a formula I compound.

As mentioned above, the present invention relates to a method for inhibiting phosphatidylinositol 3-kinase in a lysed or whole cell comprising contacting said lysed or whole cell with a compound of formula I



wherein

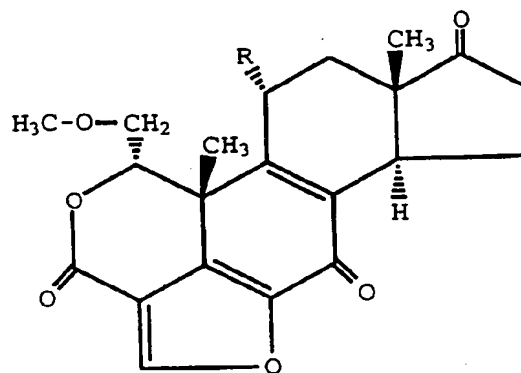
R is H or acetoxy;

R¹ is C₁-C₈ alkyl;

R² is hydrogen, C₁-C₈ alkyl, or a group of the formula -COOR³; and

R³ is H or C₁-C₈ alkyl.

The compounds of formula I generally are known in the art, and are derived from wortmannin (formula II in which R is acetoxy) or 11-desacetoxy wortmannin (formula II in which R is H).



formula II

The biosynthetic production of wortmannin is well known in the art. Typically, it is produced by the fermentation of any one of a number of previously disclosed microorganisms such as *Talaromyces wortmannii* [Nakanishi, *et al.*, *J. Biol. Chem.*, **267** (4): 2157-2163 (1992)]; and *Penicillium wortmannii*, *Myrothecium roridium*, and *Fusarium oxysporum* [Abbas, *et al.*, *Appl. Environ. Microbiol.*, **54**(5): 1267-1274 (1988)]. Following fermentation, wortmannin is extracted and purified via known methods.

Preferably, wortmannin is microbially synthesized and isolated in substantially pure form from a fermentation culture of *Penicillium duciaxii* identified as A24603.1.

Culture A24603.1 has been deposited in compliance with the Budapest Treaty, and made part of the stock culture collection of the Midwest Area Northern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 1815 North University Street, Peoria, Illinois, 61604, from which it has been assigned the accession number NRRL 21122.

The permanency of the deposit of this culture at the Midwest Area Northern Regional Research Center at Peoria, Illinois, and ready accessibility thereto by the public is afforded throughout the effective life of the patent in the event the patent is granted. Access to the culture is available during pendency of the application under 37 C.F.R. §1.14 and 35 U.S.C. §112. All restrictions on the availability to the public of the culture will be irrevocably removed upon granting of the patent.

Wortmannin is produced by culturing the above-referenced A24603.1 strain under submerged aerobic conditions in a suitable culture medium until a recoverable amount of wortmannin is produced. Wortmannin can be recovered using various isolation and purification procedures understood in the art.

The medium used to grow the A24603.1 culture can be any one of a number of media. For economy in production, optimal yield, and ease of product isolation, however, preferred carbon sources in large-scale fermentation are glucose and soluble starch such as corn starch. Maltose, ribose, xylose, fructose, galactose, mannose, mannitol, potato dextrin, methyl oleate, oils such as soybean oil and the like can also be used.

Preferred nitrogen sources are enzyme-hydrolyzed casein and cottonseed flour, although pepsinized milk, digested soybean meal, fish meal, corn steep liquor, yeast extract, acidhydrolyzed casein, beef extract, and the like can also be used.

Among the nutrient inorganic salts which can be incorporated in the culture media are the customary soluble salts capable of yielding calcium, magnesium, sodium, ammonium, chloride, carbonate, sulfate, nitrate, zinc, and like ions.

Essential trace elements necessary for the growth and development of the organism also should be included in the culture medium. Such trace elements commonly occur as impurities in other constituents of the medium in amounts sufficient to meet the growth requirements of the organism.

For production of substantial quantities of wortmannin, submerged aerobic fermentation in stirred bioreactors is preferred. Small quantities of wortmannin may be obtained by shake-flask culture. Because of the time-lag in production commonly associated with inoculation of large bioreactors with the spore form of the organism, it is preferable to use vegetative inoculum. The vegetative inoculum is prepared by inoculating a small volume of culture medium with the spore form or mycelial fragments of the organism to obtain a fresh, actively growing culture of the organism. The vegetative inoculum medium can be the same as that used for larger fermentations, but other media are also suitable.

Wortmannin is produced by the A24603.1 organism when grown at temperatures between about 23° and 29°C. Optimum temperature for wortmannin production appears to be about 25° C.

As is customary in submerged aerobic culture processes, sterile air is blown into the vessels from the bottom while the medium is stirred with conventional turbine impellers. In general, the aeration rate and agitation rate should be sufficient to maintain a level of dissolved oxygen of at least 45% of air saturation with an internal vessel pressure of about 5 atmospheres.

5 Production of wortmannin can be observed during the fermentation by testing PI 3-kinase extracts from the broth. A PI 3-kinase assay system described *infra* is a useful assay for this purpose.

Following its production, wortmannin can be recovered from the fermentation medium by methods used in the art. The wortmannin produced during fermentation of the A24603.1 organism occurs mainly in the broth.

10 Typically, wortmannin can be recovered from the biomass by a variety of techniques. A preferred technique involves filtering whole fermentation broth with a ceramic filter. The filtrate is eluted with an organic solvent such as ethylacetate and concentrated. The concentrate is suspended in alcohol until crystallization occurs and the solution is filtered, washed and dried. For confirmation, the crystalline material is dissolved in an organic solvent and chromatographed on a reverse-phase silica gel absorbent (C_8 or C_{18}). Fractions are eluted in an organic-aqueous buffer such as 60% acetonitrile.

15 From wortmannin, 17 β -hydroxywortmannin, a compound of formula I in which R is acetoxy, R¹ is methyl and R² is H, can be prepared by using procedures known in the art. Preferably, wortmannin is reduced by adding a reducing agent, particularly a metal hydride such as diisobutylaluminum hydride, to a solution of wortmannin in a suitable solvent. Suitable solvents include any solvent or mixture of solvents which will remain inert under reduction conditions.

20 In addition, 17 β -hydroxywortmannin can be further alkylated at the R¹ position via removal of the R¹ methyl group and realkylation and/or it can be alkylated at the R² position (wherein R¹ and/or R² is C₁-C₆ alkyl). 17 β -Hydroxywortmannin also can be carbonated at the R² position wherein R² is a group of the formula -COOR³ and R³ is H or C₁-C₆ alkyl. Each of these processes, dealkylation of the R¹ methyl group, realkylation of the R¹ position, alkylation of the R² position, and carbonation of the R² position can be accomplished using procedures which are well known to one of ordinary skill in the organic chemical art. For an example of preparation of steroidal carbonates, see, e.g., Ott, A.C., *J. Am. Chem. Soc.*, 74:1239 (1952).

The term "C₁-C₆ alkyl" refers to the straight or branched aliphatic chains of 1 to 6 carbon atoms including, for example, methyl, ethyl, propyl, isopropyl, butyl, n-butyl, pentyl, isopentyl, hexyl, n-hexyl, and the like.

30 11-desacetoxywortmannin (formula II in which R is H) also is known in the art as are methods for its preparation. Generally, this compound can be biosynthetically produced by fermenting a culture of *Penicillium funiculosum* Thom [see, e.g., Baggolini, *et al.*, *Exp. Cell Res.*, 169: 408-418 (1987)]; but, preferably, is chemically derived from wortmannin by the method disclosed by Haeflinger, *et al.*, *Helv. Chem. Acta*, 56(8): 2901-2904 (1973).

35 11-desacetoxy-17 β -hydroxywortmannin is prepared by reducing 11-desacetoxywortmannin via the above-described procedure for reducing wortmannin, or more particularly, as described below in the examples. 11-desacetoxy-17 β -hydroxywortmannin also can be alkylated or carbonated as described above.

40 In the present method, compounds of formula I are effective for selectively inhibiting phosphatidylinositol 3-kinase in a lysed or whole cell. This method can be carried out *in vitro* or *in vivo* and can be utilized as a pharmacological tool for studying, for example, the involvement of PI 3-kinase in mitogenesis, cellular proliferation, or cellular differentiation. The compounds of formula I also can be radiolabeled (e.g., tritiated), to provide for easier detection of such compounds in cells.

45 When a compound of formula I is used for this method, such a compound is dissolved in an organic solvent such as dimethylsulfoxide (DMSO), and diluted with HEPES buffer (pH 7.5, containing 15 mM of MgCl₂ and 1 mM of EGTA), to the desired concentration. The resulting preparation is then placed in contact with purified PI 3-kinase or a cell according to methods well known in the art.

Another embodiment of the present invention provides a method for inhibiting phosphatidylinositol 3-kinase in a mammal, particularly humans, comprising administering to said mammals a phosphatidylinositol 3-kinase inhibiting amount of a compound of formula I.

50 A preferred embodiment of the present invention includes a method for treating a phosphatidylinositol 3-kinase-dependent condition in a mammal comprising administering to said mammal a phosphatidylinositol 3-kinase inhibiting amount of a compound of formula I. PI 3-kinase-dependent conditions include biochemical processes relevant to pain, diabetes, inflammation, platelet aggregation, vascular diseases such as atherosclerosis, restenosis, and the like, and, particularly, abnormal cell growth as found in neoplasms.

55 Thus, an especially preferred embodiment of the present invention includes a method of treating phosphatidylinositol 3-kinase-dependent neoplasms, particularly various lymphosarcomas, with a compound of formula I. Other PI 3-kinase-dependent neoplasms include, for example, adenocarcinoma of the female breast, colon cancer, epidermid cancers of the head and neck, leukemia, melanoma, ovarian carcinoma, plasma cell myeloma, and squamous or small-cell lung cancer.

For therapeutic treatment of the specified indications, a compound of formula I may be administered as such, or can be compounded and formulated into pharmaceutical compositions in unit dosage form for parenteral, transdermal, rectal, nasal or intravenous administration or, preferably, oral administration. Such pharmaceutical compositions are prepared in a manner well known in the art and comprise at least one active formula I compound associated with a pharmaceutically carrier. The term "active compound", as used throughout this specification, refers to at least one formula I compound.

In such a composition, the active compound is known as "active ingredients". In making the compositions, the active ingredient usually will be mixed with a carrier, or diluted by a carrier, or enclosed within a carrier which may be in the form of a capsule, sachet, paper or other container. When the carrier serves as a diluent, it may be a solid, semisolid, or liquid material which acts as a vehicle, excipient or medium for the active ingredient. Thus, the composition can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, emulsions, solutions, syrups, suspensions, soft and hard gelatin capsules, sterile injectable solutions, and sterile packaged powders.

Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate alginates, calcium salicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, tragacanth, gelatin, syrup, methyl cellulose, methyl and propylhydroxybenzoates, talc, magnesium stearate, water, and mineral oil. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide quick, sustained, or delayed release of the active ingredient after administration to the patient by employing procedures well known in the art.

For oral administration, a compound can be admixed with carriers and diluents, molded into tablets, or enclosed in gelatin capsules. The mixtures can alternatively be dissolved in liquids such as 10% aqueous glucose solution, isotonic saline, sterile water, or the like, and administered intravenously or by injection.

The compositions preferably are formulated in a unit dosage form, each dosage containing from about 1 to about 500 mg and, more frequently, from about 5 to about 300 mg of the active ingredient. The term "unit dosage form" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with the required pharmaceutically acceptable carrier. By "pharmaceutically acceptable", it is meant the carrier, diluent or excipient must be compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The following formulation examples are illustrative only and are not intended to limit the scope of the invention in any way. The meaning of the term "active ingredient" is as defined above.

Formulation 1

Hard gelatin capsules are prepared using the following ingredients:

| | Quantity (mg/capsule) |
|--------------------|-----------------------|
| Active ingredient | 250 |
| Starch, dried | 200 |
| Magnesium stearate | 10 |
| Total | 460 mg |

Formulation 2

A tablet is prepared using the ingredients below:

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| | Quantity (mg/capsule) |
|-----------------------------|-----------------------|
| Active ingredient | 250 |
| Cellulose, microcrystalline | 400 |
| Silicon dioxide, fumed | 10 |
| Stearic acid | 5 |
| Total | 665 mg |

The components are blended and compressed to form tablets each weighing 665 mg.

Formulation 3

An aerosol solution is prepared containing the following components:

| | Percent |
|---------------------------------------|---------|
| Active ingredient | 0.25 |
| Ethanol | 25.75 |
| Propellant 22 (Chlorodifluoromethane) | 70.00 |
| Total | 100.00 |

The active compound is mixed with ethanol and the mixture added to a portion of the propellant 22, cooled to -30°C and transferred to a filling device. The required amount is then fed to a stainless steel container and diluted with the remainder of the propellant. The valve units are then fitted to the container.

Formulation 4

Tablets, each containing 60 mg of active ingredient, are made as follows:

| | Quantity (mg/tablet) |
|---|----------------------|
| Active ingredient | 60 mg |
| Starch | 45 mg |
| Microcrystalline cellulose | 35 mg |
| Polyvinylpyrrolidone (as 10% solution in water) | 4 mg |
| Sodium carboxymethyl starch | 4.5 mg |
| Magnesium stearate | 0.5 mg |
| Talc | 1 mg |
| Total | 150 mg |

The active ingredient, starch and cellulose are passed through a No. 45 mesh U.S. sieve and mixed thoroughly. The aqueous solution containing polyvinyl- pyrrolidone is mixed with the resultant powder, and the mixture then is passed through a No. 14 mesh U.S. sieve. The granules so produced are dried at 50°C and passed through a No. 18 mesh U.S. Sieve. The sodium carboxymethyl starch, magnesium stearate and talc, previously passed through a No. 60 mesh U.S. sieve, are then added to the granules which, after mixing, are compressed on a tablet machine to yield tablets each weighing 150 mg.

Formulation 5

Capsules, each containing 80 mg of active ingredient, are made as follows:

| | Quantity (mg/capsule) |
|----------------------------|-----------------------|
| Active ingredient | 80 mg |
| Starch | 59 mg |
| Microcrystalline cellulose | 59 mg |
| Magnesium stearate | 2 mg |
| Total | 200 mg |

The active ingredient, cellulose, starch, and magnesium stearate are blended, passed through a No. 45 mesh U.S. sieve, and filled into hard gelatin capsules in 200 mg quantities.

Formulation 6

Suppositories, each containing 225 mg of active ingredient, are made as follows:

| | Quantity (mg/unit) |
|---------------------------------|--------------------|
| Active ingredient | 225 mg |
| Saturated fatty acid glycerides | 2,000 mg |
| Total | 2,225 mg |

The active ingredient is passed through a No. 60 mesh U.S. sieve and suspended in the saturated fatty acid glycerides previously melted using the minimum heat necessary. The mixture is then poured into a suppository mold of nominal 2 g capacity and allowed to cool.

Formulation 7

Suspensions, each containing 50 mg of active ingredient per 5 ml dose, are made as follows:

| | Quantity |
|--------------------------------|----------|
| Active ingredient(s) | 50 mg |
| Sodium carboxymethyl cellulose | 50 mg |
| Syrup | 1.25 mL |
| Benzoic acid solution | 0.10 mL |
| Flavor | q.v. |
| Color | q.v. |
| Purified water to total | 5 mL |

The active ingredient is passed through a No. 45 mesh U.S. sieve and mixed with the sodium carboxymethyl cellulose and syrup to form a smooth paste. The benzoic acid solution, flavor and color are diluted with a portion of the water and added, with stirring. Sufficient water is then added to produce the required volume.

Formulation 8

An intravenous formulation may be prepared as follows:

| | Quantity |
|-------------------|----------|
| Active ingredient | 100 mg |
| Isotonic saline | 1,000 mL |

5

Compounds of formula I are effective against PI 3-kinase and PI 3-kinase-dependent conditions over a wide dosage range. For example, daily dosages will normally fall within the range of about 0.1 mg/kg to about 50 mg/kg of body weight. In the treatment of adult humans, the dosage range from about 5 mg/kg to about 25 mg/kg, in single or divided doses, is preferred. However, it will be understood that the amount of the compound actually administered will be determined by a physician in light of the relevant circumstances including the relative severity of a disease state, the choice of compound to be administered, the age, weight, and response of the individual patient, and the chosen route of administration. Therefore, the above dosage ranges are not intended to limit the scope of this invention in any way.

Compounds of formula I have demonstrated selective activity against PI 3-kinase. The following is a description of the test systems used to demonstrate this activity.

PI 3-kinase may be prepared by multiple methods. In one method, PI 3-kinase was prepared from confluent Swiss 3T3 cells obtained from the American Type Culture Collection, Rockville, MD. Prior to purification of PI 3-kinase, cells were maintained in bulk culture in Dulbecco's Modified Eagles Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal calf serum and were passaged using 0.25% trypsin and 0.02% ethylenediaminetetracetic acid (EDTA). 24 x 10⁶ cells on four, 100 mm culture plates were washed with 10 mL Hanks Balanced Salt Solution (HBSS; Sigma) pH 7.4, and the cells were left in DMEM without fetal calf serum for 1 hour before being stimulated for 15 minutes with 100 ng/mL of the recombinant human BB homodimer of platelet derived growth factor (PDGF; Genzyme, Cambridge, MA). The medium was aspirated and the cells washed with 10 mL of HBSS before being lysed with 3 mL of 137 mM NaCl, 20 mM of Tris (pH 8.0) containing 1 mM of MgCl₂, 10% of glycerol, 1% of Triton X-100 (Rohm and Haas, Philadelphia, PA), 2 µg/mL of leupeptin, 2 µg/mL of aprotinin, 1 mM of phenylmethylsulfonyl fluoride (PMSF), and 1 mM of sodium orthovanadate. The cells were scraped free from the surface of the dish and centrifuged at 6,000 x g for 10 minutes. The supernatant was mixed with 50 µL of washed IgG2bk antiphosphotyrosine antibody beads (Upstate Biotechnology Inc., Lake Placid, NY) in 1.5 mL tubes. The tubes were capped and rotated for 2 hours at 4° C and the beads were twice washed with 1 mL of HBSS containing 2 µg/mL of leupeptin, 4 µg/mL of aprotinin, 1 mM of PMSF, 200 µM of adenosine, and 1 mM of sodium orthovanadate. The tyrosine phosphorylated PI 3-kinase was eluted from the beads with 200 µL/tube of 10 mM Tris (pH 7.5), 2 M of NaCl, 1 mM of EDTA, 200 µM of adenosine, and 10 mM of sodium phenylphosphate.

In another, preferred, method, PI 3-kinase was prepared from bovine brain. Two bovine brains (wet weight about 900 g) were obtained from a local slaughterhouse within minutes of slaughter, packed on ice, and homogenized within one hour. Brains were trimmed of excess fat and blood vessels and then homogenized using a Tekmar Tissuermizer (Cincinnati, OH) at 4° C in 20 mM of Tris (pH 8.3) containing 250 mM of sucrose, 6 mM of β-mercaptoethanol, 1 µg/mL of leupeptin, 1 µg/mL of pepstatin A, 0.4 mM of PMSF, and 1 mM of MgCl₂.

Following centrifugation for 60 minutes at 10,000 x g, the pH of the supernatant (about 1200 mL) was lowered to 5.75 using dropwise addition of 1M acetic acid at 4° C. After stirring for an additional 15 minutes at 4° C, the solution was centrifuged for 60 minutes at 13,500 x g. The supernatant was discarded. Pellets were resuspended in Buffer A (20 mM of Tris, pH 8.3, containing 6 mM of β-mercaptoethanol, 0.1 mM of ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 µg/mL of leupeptin, 1 µg/mL of pepstatin A, and 1 mM of MgCl₂), and loaded onto a Fast Flow Q Sepharose column (300 mL) at a flow rate of 5 mL/min at 4° C. After loading, the column was washed with 3 volumes of Buffer A containing 0.1 M of KCl and the kinase was then eluted with a linear gradient of Buffer A/0.1M KCl to Buffer A/0.6 M KCl at 3 mL/min over 7 volumes.

Fractions were assayed for PI 3-kinase activity using 10 µL of the fraction and phosphatidylinositol as substrate as described below. PI 4-kinase eluted in the breakthrough; PI 3-kinase eluted at approximately 0.3 M of KCl. The PI 3-kinase pool was subjected to a 40% ammonium sulfate precipitation. Following centrifugation (60 minutes at 13,500 x g), pellets were resuspended in Buffer B (10 mM of potassium phosphate, pH 7.4, containing 6 mM of β-mercaptoethanol, 1 µg/mL of leupeptin, 1 µg/mL of pepstatin A, and 1 mM of MgCl₂), and loaded onto a 50 mL hydroxylapatite column (Calbiochem, Inc., La Jolla, CA) at 2.5 mL/minute. The column was washed with 150 mL Buffer B until the A₂₈₀ baseline reached zero, and the kinase was then eluted with a linear gradient of 10-320 mM of KH₂PO₄ at 1 mL/minute over 450 minutes.

Active fractions were pooled and then loaded at 3 mL/minute onto a MonoS column (8 mL) (Pharmacia, Inc., Piscataway, NJ) equilibrated in Buffer C (50 mM of MES, pH 6.2, containing 6 mM of β-mercaptoethanol, 0.1 mM of EGTA, 1 µg/mL of leupeptin, 1 µg/mL of pepstatin A, and 1 mM of MgCl₂). PI 3-kinase was eluted

| | Quantity |
|-------------------|----------|
| Active ingredient | 100 mg |
| Isotonic saline | 1,000 mL |

5

Compounds of formula I are effective against PI 3-kinase and PI 3-kinase-dependent conditions over a wide dosage range. For example, daily dosages will normally fall within the range of about 0.1 mg/kg to about 50 mg/kg of body weight. In the treatment of adult humans, the dosage range from about 5 mg/kg to about 25 mg/kg, in single or divided doses, is preferred. However, it will be understood that the amount of the compound actually administered will be determined by a physician in light of the relevant circumstances including the relative severity of a disease state, the choice of compound to be administered, the age, weight, and response of the individual patient, and the chosen route of administration. Therefore, the above dosage ranges are not intended to limit the scope of this invention in any way.

Compounds of formula I have demonstrated selective activity against PI 3-kinase. The following is a description of the test systems used to demonstrate this activity.

PI 3-kinase may be prepared by multiple methods. In one method, PI 3-kinase was prepared from confluent Swiss 3T3 cells obtained from the American Type Culture Collection, Rockville, MD. Prior to purification of PI 3-kinase, cells were maintained in bulk culture in Dulbecco's Modified Eagles Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal calf serum and were passaged using 0.25% trypsin and 0.02% ethylenediaminetetracetic acid (EDTA). 24×10^6 cells on four, 100 mm culture plates were washed with 10 mL Hanks Balanced Salt Solution (HBSS; Sigma) pH 7.4, and the cells were left in DMEM without fetal calf serum for 1 hour before being stimulated for 15 minutes with 100 ng/mL of the recombinant human BB homodimer of platelet derived growth factor (PDGF; Genzyme, Cambridge, MA). The medium was aspirated and the cells washed with 10 mL of HBSS before being lysed with 3 mL of 137 mM NaCl, 20 mM of Tris (pH 8.0) containing 1 mM of $MgCl_2$, 10% of glycerol, 1% of Triton X-100 (Rohm and Haas, Philadelphia, PA), 2 μ g/mL of leupeptin, 2 μ g/mL of aprotinin, 1 mM of phenylmethyl-sulfonyl fluoride (PMSF), and 1 mM of sodium orthovanadate. The cells were scraped free from the surface of the dish and centrifuged at $6,000 \times g$ for 10 minutes. The supernatant was mixed with 50 μ L of washed IgG2bk antiphosphotyrosine antibody beads (Upstate Biotechnology Inc., Lake Placid, NY) in 1.5 mL tubes. The tubes were capped and rotated for 2 hours at $4^\circ C$ and the beads were twice washed with 1 mL of HBSS containing 2 μ g/mL of leupeptin, 4 μ g/mL of aprotinin, 1 mM of PMSF, 200 μ M of adenosine, and 1 mM of sodium orthovanadate. The tyrosine phosphorylated PI 3-kinase was eluted from the beads with 200 μ L/tube of 10 mM Tris (pH 7.5), 2 M of NaCl, 1 mM of EDTA, 200 μ M of adenosine, and 10 mM of sodium phenylphosphate.

In another, preferred, method, PI 3-kinase was prepared from bovine brain. Two bovine brains (wet weight about 900 g) were obtained from a local slaughterhouse within minutes of slaughter, packed on ice, and homogenized within one hour. Brains were trimmed of excess fat and blood vessels and then homogenized using a Tekmar Tissuemizer (Cincinnati, OH) at $4^\circ C$ in 20 mM of Tris (pH 8.3) containing 250 mM of sucrose, 6 mM of β -mercaptoethanol, 1 μ g/mL of leupeptin, 1 μ g/mL of pepstatin A, 0.4 mM of PMSF, and 1 mM of $MgCl_2$.

Following centrifugation for 60 minutes at $10,000 \times g$, the pH of the supernatant (about 1200 mL) was lowered to 5.75 using dropwise addition of 1M acetic acid at $4^\circ C$. After stirring for an additional 15 minutes at $4^\circ C$, the solution was centrifuged for 60 minutes at $13,500 \times g$. The supernatant was discarded. Pellets were resuspended in Buffer A (20 mM of Tris, pH 8.3, containing 6 mM of β -mercaptoethanol, 0.1 mM of ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 μ g/mL of leupeptin, 1 μ g/mL of pepstatin A, and 1 mM of $MgCl_2$), and loaded onto a Fast Flow Q Sepharose column (300 mL) at a flow rate of 5 mL/min at $4^\circ C$. After loading, the column was washed with 3 volumes of Buffer A containing 0.1 M of KCl and the kinase was then eluted with a linear gradient of Buffer A/0.1M KCl to Buffer A/0.6 M KCl at 3 mL/min over 7 volumes.

Fractions were assayed for PI 3-kinase activity using 10 μ L of the fraction and phosphatidylinositol as substrate as described below. PI 4-kinase eluted in the breakthrough; PI 3-kinase eluted at approximately 0.3 M of KCl. The PI 3-kinase pool was subjected to a 40% ammonium sulfate precipitation. Following centrifugation (60 minutes at $13,500 \times g$), pellets were resuspended in Buffer B (10 mM of potassium phosphate, pH 7.4, containing 6 mM of β -mercaptoethanol, 1 μ g/mL of leupeptin, 1 μ g/mL of pepstatin A, and 1 mM of $MgCl_2$), and loaded onto a 50 mL hydroxylapatite column (Calbiochem, Inc., La Jolla, CA) at 2.5 mL/minute. The column was washed with 150 mL Buffer B until the A_{280} baseline reached zero, and the kinase was then eluted with a linear gradient of 10-320 mM of KH_2PO_4 at 1 mL/minute over 450 minutes.

Active fractions were pooled and then loaded at 3 mL/minute onto a MonoS column (8 mL) (Pharmacia, Inc., Piscataway, NJ) equilibrated in Buffer C (50 mM of MES, pH 6.2, containing 6 mM of β -mercaptoethanol, 0.1 mM of EGTA, 1 μ g/mL of leupeptin, 1 μ g/mL of pepstatin A, and 1 mM of $MgCl_2$). PI 3-kinase was eluted

with a linear gradient of 0-0.4 M KCl in Buffer C over 120 minutes. In assaying fractions, two pools of PI 3-kinase activity were routinely found. The bulk of the activity was found in the flow-through, while about 20% of the activity was eluted in the gradient. Although the material in the gradient had considerable PI 4-kinase activity, essentially no PI 4-kinase activity was associated with the PI 3-kinase eluted in the flow-through. Therefore, the MonoS flow-through was concentrated by tangential flow filtration on a Mini-Ultrasette Omega 50 K membrane (Filtron, Inc., Northborough, MA) and diluted in Buffer C to lower the conductivity. The material was then reloaded onto the MonoS column using the above conditions. The PI 3-kinase bound to the column during the wash and was eluted in the gradient. Two pools of phosphatidylinositol kinase activity were obtained in the gradient; each was assayed for PI 3-kinase and PI 4-kinase activity. Pool I was found to contain 95% PI 3-kinase activity (and 5% PI 4-kinase) while Pool II contained predominantly PI 4-kinase activity.

Pool I from the MonoS column was diluted with Buffer A and chromatographed on MonoQ (1 ml) and eluted with a gradient of 0-0.4 M KCl in Buffer A. The final pool was assayed for PI 3-kinase and PI 4-kinase activity. The final product was found to contain greater than 99% PI 3-kinase activity.

PI 3-kinase activity was measured as previously described by Matter, W.F., *et al.*, Biochemical and Biophysical Research Communications, **186**: 624-631 (1992). Inhibitor candidates were initially dissolved in DMSO and then diluted 10-fold with 50 mM of HEPES buffer, pH 7.5, containing 15 mM of $MgCl_2$ and 1 mM of EGTA. Ten microliters of this solution were incubated with purified bovine brain PI 3-kinase (9 μ L) and phosphatidylinositol (5 μ L of a 2 mg/mL stock solution in 50 mM of HEPES buffer, pH 7.5, containing 1 mM of EGTA). The final reaction mixture contained 0.1-5 ng/mL of inhibitor and 3% of DMSO (v:v). This concentration of DMSO had no effect on PI 3-kinase activity; control reaction mixtures contained 3% of DMSO (v:v) without inhibitor. Reactants were preincubated 10 minutes at ambient temperature and then the enzyme reaction was started upon addition of 1 μ L [γ - ^{32}P]ATP (2 mCi/mL, 500 μ M of stock solution; 0.08 mCi/mL, 20 μ M of final concentration; Dupont New England Nuclear, Boston, MA). The reaction was allowed to proceed for 10 minutes at ambient temperature with frequent mixing, after which time the reaction was quenched by addition of 40 μ L of 1N HCl. Lipids were extracted with addition of 80 μ L $CHCl_3$:MeOH (1:1, v:v). The samples were mixed and centrifuged, and the lower organic phase was applied to a silica gel TLC plate (EM Science, Gibbstown, NJ), which was developed in $CHCl_3$:MeOH:H₂O:NH₄OH (45:35:8.5:1.5, v:v). Plates were dried, and the kinase reaction visualized by autoradiography. The phosphatidylinositol 3-monophosphate region was scraped from the plate and quantitated using liquid scintillation spectroscopy with ReadyProtein (Beckman Instruments, Inc., Fullerton, CA) used as the scintillation cocktail. The level of inhibition for wortmannin and analogs was determined as the percentage of [^{32}P]-counts per minute compared to controls.

Alternatively, products of the PI 3-kinase reaction were confirmed by HPLC as discussed by Whitman, M., Nature, **332**: 644-646 (1988). Phospholipids were deacylated in methylamine reagent and separated using a Whatman Partisphere SAX anion exchange column as previously described by Auger, K.R., Cell, **57**: 167-175 (1989). A Radiomatic Model A-140 Flo-One/Beta on-line radioactivity detector was used to monitor the deacylated [^{32}P]-enzyme products; deacylated [3H]PI 4-monophosphate was added as an internal standard.

When tested on bovine brain purified PI 3-kinase, 17 β -hydroxywortmannin, an exceptional IC_{50} of 0.2 ng/ml (0.46 nM) was recorded. In addition, 17 β -hydroxywortmannin has no effect on PI 4-kinase, phospholipase C, c-src protein tyrosine kinase or protein kinase C, and have a potency up to one hundred-fold greater than the previously reported activity as an inhibitor of myosin light chain kinase [see, e.g., Nakanishi, S., *et al.*, J. Biol. Chem., **267**(4):2157-2163 (1992)]. Thus, the compounds used in the methods of the present invention, particularly 17 β -dihydroxywortmannin, are potent, highly selective inhibitors of PI 3-kinase.

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood, however, that these examples are only for illustrative purposes and are not to be construed as limiting the scope of this invention in any manner.

Example 1

Fermentation of Culture A24603.1

A. Shake-Flask

The culture A24603.1, either as a lyophilized pellet or as a suspension maintained in liquid nitrogen, is used to inoculate a vegetative medium having the following composition

Vegetative Medium

| | <u>Ingredient</u> | <u>Amount (g/L)</u> |
|----|-------------------------------|---------------------|
| 5 | Glucose | 10.0 |
| | Glycerol | 10.0 |
| 10 | Cottonseed Flour ^a | 25.0 |

Unadjusted pH=6.3; no adjustment

15 ^a PROFLO Flour (Traders Protein, Memphis, TN).

20 The inoculated vegetative medium was incubated in a 250 mL wide-mouth Erlenmeyer flask at 25° C for about 72 hours on a shaker orbiting in a two-inch (5.08 cm) circle at 250 rpm.

B. Tank Fermentation of Culture A24603.1

25 In order to provide a larger volume of inoculum, 10 mL of incubated shake-flask medium, prepared as described in Section A, was used to inoculate 400 mL of a second-stage vegetative medium having the same composition as described above. This second-stage medium was incubated in a 2-L wide-mouth Erlenmeyer flask at 25° C for about 23 hours on a shaker orbiting in a two-inch (5.08 cm) circle at 250 rpm.

30 This second-stage medium (400 mL) was used to inoculate 115 L of sterile production medium having the following composition.

Production Medium

| | <u>Ingredient</u> | <u>Amount (g/L)</u> |
|----|-------------------------------|---------------------|
| 5 | Glucose | 25.0 |
| | Corn Starch | 10.0 |
| 10 | Lexein | 10.0 |
| | Enzyme-hydrolyzed casein | 4.0 |
| 15 | Blackstrap molasses | 5.0 |
| | MgSO ₄ (anhydrous) | 5.0 |
| | CaCO ₃ | 2.0 |
| 20 | Deionized H ₂ O | q.s. to 115 L |

Unadjusted pH = 6.8; no adjustment.

Antifoam agent added: SAG 471^b (0.2 gm/L).

^a NZ Amine A (Sheffield Chemical Co., Norwich, NY).

^b SAG 471 (Union Carbide, Sistersville, WV).

The inoculated production medium was allowed to ferment in a 115-L stirred fermentation tank for 4-5 days at a temperature of about 25° C. A dissolved oxygen level of about 45% of air saturation was maintained, as was a low rpm (180-330) in the stirred vessel.

Example 2Isolation and Purification of Wortmannin

Fermentation broth from Example 1 was filtered through a ceramic filter (Membralox Systems, Illinois Water Treatment, Rockford, IL) to yield 175 L of filtrate containing wortmannin. The pH of the filtrate was adjusted to about 3.9 with 5N HCl. The filtrate was then eluted three times with one-half volumes of ethyl acetate to give a combined volume of 207 L which was concentrated to 6 L *in vacuo*.

The 6 L of ethyl acetate concentrate was further concentrated *in vacuo* to form a dark brown viscous oil to which 500 mL of methanol was added. The mixture was swirled until the resulting crystallization was complete, filtered, briefly washed with cold methanol and dried *in vacuo* to give 20.4 g of wortmannin.

The methanol supernatant was reconstituted *in vacuo* to form a viscous oil, dissolved in 180 mL of chloroform and applied to a 12 x 20 cm column of Woelm Grade 62 silica in chloroform. 5.0 L of chloroform wash was concentrated *in vacuo* to form a brown oil which was then dissolved in 250 mL of warm methanol. The resulting crystals were collected after 18 hours, via filtration, giving 4.2 g of wortmannin. The crystallization procedure was repeated on the remaining supernatant, yielding an additional 1.9 g of wortmannin. The identity of wortmannin was confirmed by HPLC.

Example 317 β -hydroxywortmannin

To a solution of wortmannin (100 mg) stirring in THF at -78° C was added diisobutylaluminum hydride (0.4

Production Medium

| | <u>Ingredient</u> | <u>Amount (g/L)</u> |
|----|-------------------------------|---------------------|
| 5 | Glucose | 25.0 |
| | Corn Starch | 10.0 |
| 10 | Lexein | 10.0 |
| | Enzyme-hydrolyzed casein | 4.0 |
| 15 | Blackstrap molasses | 5.0 |
| | MgSO ₄ (anhydrous) | 5.0 |
| | CaCO ₃ | 2.0 |
| 20 | Deionized H ₂ O | q.s. to 115 L |

Unadjusted pH = 6.8; no adjustment.

25 Antifoam agent added: SAG 471^b (0.2 gm/L).

^a NZ Amine A (Sheffield Chemical Co., Norwich, NY).

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The inoculated production medium was allowed to ferment in a 115-L stirred fermentation tank for 4-5 days at a temperature of about 25° C. A dissolved oxygen level of about 45% of air saturation was maintained, as was a low rpm (180-330) in the stirred vessel.

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45 The 6 L of ethyl acetate concentrate was further concentrated *in vacuo* to form a dark brown viscous oil to which 500 mL of methanol was added. The mixture was swirled until the resulting crystallization was complete, filtered, briefly washed with cold methanol and dried *in vacuo* to give 20.4 g of wortmannin.

50 The methanol supernatant was reconcentrated *in vacuo* to form a viscous oil, dissolved in 180 mL of chloroform and applied to a 12 x 20 cm column of Woelm Grade 62 silica in chloroform. 5.0 L of chloroform wash was concentrated *in vacuo* to form a brown oil which was then dissolved in 250 mL of warm methanol. The resulting crystals were collected after 18 hours, via filtration, giving 4.2 g of wortmannin. The crystallization procedure was repeated on the remaining supernatant, yielding an additional 1.9 g of wortmannin. The identity of wortmannin was confirmed by HPLC.

55 Example 317 β -hydroxywortmannin

To a solution of wortmannin (100 mg) stirring in THF at -78° C was added diisobutylaluminum hydride (0.4

mL of a 1.0 M solution in toluene, 0.4 mmol). After 0.5 hour, the reaction was quenched with saturated aqueous NaHCO₃. The mixture was then warmed to room temperature and extracted with CH₂Cl₂. The combined organic extracts were washed with brine and dried (MgSO₄). The crude material was purified by radial chromatography (SiO₂, 4 mm, 9:1 EtOAc/Hexanes) to give 17β-hydroxywortmannin as an off-white powder.

¹H NMR (300 MHz, CDCl₃) 8.22 (s, 1H), 6.10 (m, 1H), 4.76 (dd, 1H), 3.88 (s, 1H), 3.44 (dd, 1H), 3.20 (s, 3H), 2.95 (1/2 ABq, 1H), 2.75 (m, 1H), 2.65 (1/2 ABq, 1H), 2.52 (m, 1H), 2.10-2.30 (m, 4H), 1.4-1.7 (m), 0.85 (2, 3H), FD MS M⁺ 431, IR (Cell, CDCl₃), 1751, 1680 cm⁻¹).

Example 4

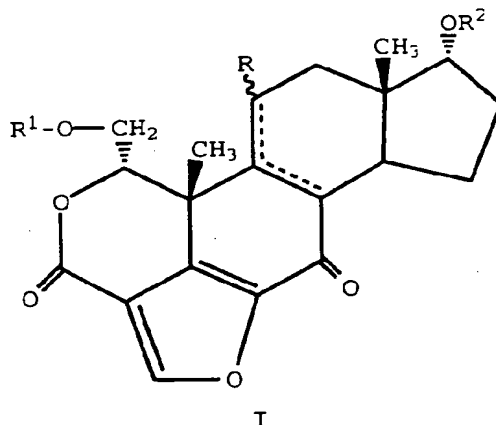
11-Desacetoxy-17β-hydroxywortmannin

To a solution of 11-desacetoxywortmannin [prepared via the method of Haeflinger, W.; Hauser, D., *Helv. Chim. Acta*, **56**, 2901, (1973)] stirring in THF at -78° C was added diisobutylaluminum hydride (0.1 mL of a 1.0 M solution in toluene). After 1 hour, the reaction was quenched with saturated aqueous NaHCO₃. The mixture was then warmed to room temperature and extracted with CH₂Cl₂. The combined organic extracts were washed with brine and dried (MgSO₄). The crude material was purified by radial chromatography (SiO₂, 1 mm, 9:1 EtOAc/Hexanes) to give the titled product as a tan powder.

¹H-NMR (300 MHz, CDCl₃) 8.19 (s, 1H), 4.81 (s, 1H), 3.80 (t, 1H), 3.15 (s, 3H), 1.7 (s, 3H), 0.7 (s, 3H). FAB MS (M⁺H)⁺ 373.3.

Claims

1. Use of a compound of formula I



wherein

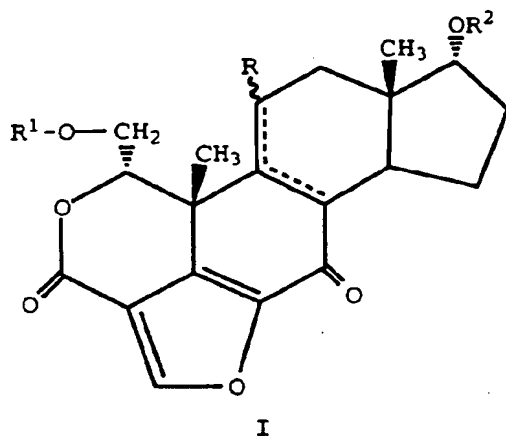
R is H or acetoxy;

R¹ is C₁-C₆ alkyl;

R² is hydrogen, C₁-C₆ alkyl, or a group of the formula -COOR³; and

R³ is H or C₁-C₆ alkyl, for the manufacture of a medicament for inhibiting phosphatidylinositol 3-kinase in mammals.

2. The use according to Claim 1 wherein said compound is a compound wherein R¹ is methyl and R² is H.
3. The use according to Claim 2 wherein said compound is a compound wherein R is H.
4. The use according to Claim 2 wherein said compound is a compound wherein R is acetoxy.
5. Use of a compound of formula I



wherein

R is H or acetoxy;

R¹ is C₁-C₈ alkyl;

R² is hydrogen, C₁-C₈ alkyl, or a group of the formula -COOR³; and

R³ is H or C₁-C₈ alkyl, for the manufacture of a medicament for treating phosphatidylinositol 3-kinase-dependent conditions in mammals.

6. The use according to Claim 5 wherein said phosphatidylinositol 3-kinase-dependent condition is a neoplasm.
7. The use according to Claim 6 wherein said compound is a compound wherein R¹ is methyl and R² is H.
8. The use according to Claim 7 wherein said compound is a compound wherein R is H.
9. The use according to Claim 7 wherein said compound is a compound wherein R is acetoxy.



European Patent
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EUROPEAN SEARCH REPORT

Application Number
EP 94 30 6124

| DOCUMENTS CONSIDERED TO BE RELEVANT | | | |
|--|---|--|---|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.6) |
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| Y | * abstract * * page 415 - page 417 * | 1-4,6,8 | |
| P,Y | J.BIOL.CHEM., vol.268, no.34, 5 December 1993 pages 25846 - 25856 HIROSHI YANO ET AL. 'Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells' * abstract * | 1-4 | |
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| The present search report has been drawn up for all claims | | | |
| Place of search MUNICH | | Date of completion of the search 19 December 1994 | Examiner Tzschoppe, D |
| <p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document</p> | | | |

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EUROPEAN SEARCH REPORT

Application Number
EP 94 30 6124

| DOCUMENTS CONSIDERED TO BE RELEVANT | | | |
|--|---|--|--|
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| The present search report has been drawn up for all claims | | | |
| Place of search MUNICH | | Date of completion of the search 19 December 1994 | Examiner Tzschoppe, D |
| <p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document</p> | | | |

EPO FORM 1501 (04/94) (P.O. 04/94)